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Permeation studies of chlorpyrifos through skin and synthetic membranes to improve the *in vitro* dermal absorption assay of lipophilic compounds with ethanolic receptors

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ABSTRACT

The *in vitro* percutaneous absorption assay is standardized, but the common use of 50 % ethanol in the receptor compartment for lipophilic compounds is questioned. In parallel, the demand for animal-free methodologies is driving the application of synthetic membranes without standardization guidelines. To address these issues, this study investigated the permeation of the lipophilic compound chlorpyrifos using different ethanol-containing receptor fluids with human and pig skin *ex vivo*, and silicone and STRAT-M® membranes. The results considered several factors, particularly chlorpyrifos solubility and the contact angles between skin models and receptor fluids. Original experimental approaches demonstrated that ethanol from the receptor rapidly crosses to the donor compartment increasing chlorpyrifos diffusivity. Compared to the described *in vivo* dermal absorption, human skin and STRAT-M® yielded the best predictive permeation parameters. However, high percentage of ethanol in the receptor fluid can lead to an overestimation of percutaneous absorption. Summing up, it is important to carefully determine the concentration of ethanol to be used in the receptor fluid of lipophilic compounds' assays while further research with synthetic membranes is needed prior to their wider adoption.

1. Introduction

The *in vitro* assay of percutaneous absorption of chemicals is widely used to investigate the dermal absorption of chemical compounds. Franz cells (Franz, 1975) and other format diffusion cells have been valuable tools for skin permeation experiments supporting risk assessment and drug delivery studies of candidate drugs, toxicants, and skin formulation excipients (Azarbayjani et al., 2010; Beriro et al., 2016; Chedik et al., 2024; OECD, 2022; Silva et al., 2021a; Sung et al., 2019; Thors et al., 2016). These experimental systems are apparatus composed of a donor and receptor compartments tightly connected with a skin sample or other permeation barrier in between. After applying the chemical to test on the external part of the skin in the donor compartment, its gradual accumulation in the fluid filling the receptor compartment indicates its ease of permeation through the skin.

There have been significant advances in the standardization of these assays. The Organization for Economic Co-operation and Development (OECD) provides guidelines for the experimental conditions (OECD, 2004, 2022) together with the calculus of two essential parameters, the flux and lag time (Tlag) of permeation that require a constant compound concentration at the skin surface over the exposure time respecting the sink condition (OECD, 2004, 2022). However, some methodological issues of the assay remain under debate, such as the appropriate skin source, vehicles and receptor fluid composition for poorly soluble analytes (Beriro et al., 2016; Chedik et al., 2024; Hopf et al., 2020).

As surrogate for human skin, pig or non-human primate skin are considered the best predictive models of human percutaneous absorption to be used in the *in vitro* assays (OECD, 2022). In parallel, a related

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Table 1
– Quantification of chlorpyrifos dermal absorption reported in the literature. Flux (or absorption rate) and lag time, besides the concentration and quantity of chlorpyrifos permeated at 8h through skin, in different conditions, are presented. The quantity of chlorpyrifos at 8h was normalized to the permeation area of the corresponding diffusion cells (or area of *in vivo* exposed skin).

Study and Dose	Flux (μg cm^{-2} h^{-1})	Lag time (h)	[Chlorpyrifos] (μg/mL)	Chlorpyrifos permeated *per* area (μg/cm^2)
(Sartorelli et al., 1998) Monkey skin *ex vivo* Permeation assays in static diffusion cells (diffusion area 1.77 cm^2) with receptor fluid containing 4 % (w/v) of BSA				
14.8 μg/cm^2	18.89×10^{-3}	7.46 ± 0.52	ND	0.175
(Griffin et al., 2000) Human skin *ex vivo* Permeation assays in flow-through diffusion cells with receptor fluid (volume 0.178 mL) containing 50 % (v/v) of ethanol				
732 μg/cm^2	1.7	0	3.9	11.7
(Moore et al., 2014) Human skin *ex vivo* Permeation assays in flow-through diffusion cells with receptor fluid (volume 0.4 mL) containing 2 % (w/v) of BSA				
11.2 μg/cm^2	$(3.1 \pm 0.2) \times 10^{-3}$	5.9 ± 1.6	0.080	0.017
(Griffin et al., 1999) Human dermal absorption *in vivo* Exposure for 8 h and determination of urinary metabolites				
400 μg/cm^2	0.456 (absorption rate)	<1h	ND	3.64

ND – No data available.

and emerging topic is the development of animal-free methodologies to replace conventional assays using biological tissues like skin. Diverse synthetic membranes have been investigated as alternatives that mimic the permeation of compounds across human skin (Haq et al., 2018; Kovács et al., 2021; Marques-da-Silva et al., 2022; Neupane et al., 2020). Silicone membrane was shown to mimic the skin permeation of caffeine (Uchida et al., 2016), and the STRAT-M® membrane was suggested as a valuable tool to investigate dermal absorption of hydrophobic and low hydrophilic compounds like plasticizers and several drugs (Merck, 2018; Olkowska and Gržinić, 2022; Simon et al., 2016; Uchida et al., 2015). In this sense, silicone and STRAT-M® membranes stand out compared to *ex vivo* skin regarding their accessibility, cost-effectiveness and easy-to-use properties. Nevertheless, the OECD guidelines do not emit any consideration regarding the possible use of these alterative barriers in assays for assessing dermal absorption of chemicals. In addition, the lack of studies of synthetic membranes with varying receptor fluids and other experimental conditions is another gap hindering their adoption in new approach methodologies.

The composition of the fluid in the receptor compartment of the diffusion cells employed in the dermal absorption assays of lipophilic compounds is one of the experimental issues that is most often emphasized for standardization (Beriro et al., 2016; Chedik et al., 2024; Hopf et al., 2020; Thors et al., 2016). While saline solution is considered appropriate for testing hydrophilic solutes, it is not suitable for hydrophobic compounds with low solubility in aqueous media. For these cases, the guidelines recommend the use of solvent mixtures, like 50 % (v/v) ethanol or 5 % (w/v) bovine serum albumin (BSA), to ensure the solubility of the tested compound in the receptor fluid does not act as a rate-limiting step in the permeation process (OECD, 2022). In addition, the SCCS Guidelines provide additional criteria on the receptor fluid such as having a physiological pH without interfering with skin integrity (SCCS, 2010). The criteria of solubility for the penetrated substance in the receptor fluid (<10 % saturation) coincides with the criteria indicated by the OECD guidelines (OECD, 2022; SCCS, 2010). However, the guidelines do not discuss a preferred option regarding receptor fluids when investigating lipophilic compounds, nor is there complete support for using ethanol at that specific percentage. Being the inclusion of ethanol in the fluid a very practical means to increase the solubility of lipophilic compounds, different authors have been using diverse concentrations of the solvent in the receptors of skin permeation assays, from 10 % to more than 50 % (Chedik et al., 2024; Haq and Michniak-Kohn, 2018). Few works investigate the effect of the concentration of ethanol, but there are indications that high percentage ethanolic receptors might overestimate the human skin permeability to compounds (Silva et al., 2021a; Thors et al., 2016). Indeed, ethanol is well established to enhance the permeation of compounds across the skin by different mechanisms (Berner et al., 1989; Bommannan et al., 1991; Gupta et al., 2020; Pershing et al., 1990; Watkinson et al., 2009).

In this work, we investigated the permeation of a lipophilic compound using ethanol-containing solutions in the receptor compartment of Franz cells, both with skin and synthetic membranes. Chlorpyrifos was selected as the test compound for several reasons. This pesticide is quite hydrophobic (log $K_{o/w}$ approximately 5), with a water solubility similar to other compounds of relevance concerning dermal absorption such as polycyclic aromatic hydrocarbons, estradiol, bisphenol A or parabens (Beriro et al., 2016; Chedik et al., 2024; Pershing et al., 1990; Silva et al., 2021a). Chlorpyrifos is a pesticide widely used and associated with concerning dermal absorption risks derived from occupational exposure (EPA, 2020; Farahat et al., 2010; Fenske and Elkner, 1990). Moreover, in a previous work we noticed that the published results from *in vitro* percutaneous absorption fluxes of chlorpyrifos measured with ethanol (50 %)- and BSA-containing receptors varied more than 2 orders of magnitude (Silva et al., 2021a). Finally, there is a significant body of chlorpyrifos biomonitoring and human dermal administration studies (Fenske and Elkner, 1990; Griffin et al., 1999; Hayat et al., 2019; Nolan et al., 1984; Panuwet et al., 2008; Waheed et al., 2017), which are summarized in Table S1 of Supplementary Information (SI). Among these works, the controlled dermal administration study by Griffin et al. (1999) provides useful *in vivo* data to compare with the *in vitro* results published (Table 1) and those herein reported.

In a nutshell, the results obtained in this work support the use of ethanolic receptors instead of BSA-containing solutions in the *in vitro* dermal permeation assay of lipophilic compounds. However, the novel experimental approaches herein implemented point out the importance of avoiding high percentage ethanolic fluids, as ethanol penetrating the skin and synthetic membranes modifies their permeability. We believe this work will contribute to leveraging research in risk assessment and dermal absorption of hydrophobic compounds – potential toxicants and drugs - in two essential directions: 1) gain a further understanding of the influence of ethanol in the receptor fluid used in the *in vitro* dermal permeation assay; 2) promote the use of alternative skin models in this type of studies.

2. Methods

2.1. Chemicals and human plasma

Chlorpyrifos was purchased from Sigma-Aldrich (purity ≥98 %, cat. no. 45395), as well as acetone (≥99.8 %, 34850). Acetonitrile and n-hexane were from Chem-Lab/Honeywell (cat. no. 34851 and 34859),

while ethanol absolute and BSA (fatty acid-free) were from Fisher Chemical (E/0650DF/C17 and BP9704-100). Sodium azide was from Riedel-de Haen (cat. no. 13412) and sodium chloride was from Biochem Chemopharma (319120500). The plasma was purchased from Biowest (cat. no. S4180, batch S00P81), and it has been processed from blood from different donors collected on citrate phosphate dextrose.

2.2. Skin and synthetic membranes

Human skin explants with a diameter between 180 mm and 200 mm and a thickness inferior to 500 μm were provided by PRIMACYT Cell Culture Technology GmbH (Schwerin, Germany) and delivered in the laboratory in dry ice. Until the permeation assays the discs were stored at −20 °C and then, on the experiment day, the pieces were thawed at room temperature. Throughout this work we used human skin from 5 different donors.

Pig skin was obtained from fresh pig ears, acquired at a local butcher's, and the dermatomed skin was excised from the external-dorsal part of the ear. Skin pieces were cut to an approximate area of 1.77 cm^2 and their thickness was measured with a micrometer for a final thickness of 500–700 μm. The skin pieces were placed in aluminum foil and stored at −20 °C for a maximum of 6 months, and on the experiment day, the pieces were thawed at room temperature. Throughout this work we used pig skin from 4 different animals.

STRAT-M® membrane was from Millipore (product SKBM02560), and the silicone membrane was provided by Lintec (product LTC S1-75). Both membranes were mounted in the Franz cells for the permeation assays without any previous treatment.

A representative photograph and more details on the skin models used can be found in Table S2 of the SI.

2.3. Chlorpyrifos permeation assays

The permeation assays were carried out following the steps described by OECD guidelines (OECD, 2004, 2022). Static diffusion cells (Franz cells - SES GmbH 4G-01-00-09-05, Permegear, Germany) were used with a permeation area of 0.64 cm^2. A schematic representation of the Franz diffusion cell can be found in Appendix A of the SI. Each skin model was mounted between the donor and receptor compartments of the cells. The human and pig skins were mounted with the epidermis facing the donor compartment, and the silicone and the STRAT-M® membranes were mounted with the shiny section facing the donor compartment. For every skin model, after mounting it in the donor compartment, the watertight conditions were tested as a quality control test. In the case of pig skin, the integrity of the batches used in each assay was regularly checked by standard assays with the reference compound caffeine (OECD, 2022). In cases where the result of the permeability coefficient was 100x higher or lower than the accepted value, 3.0×10^{-8} cm/s (Silva et al., 2021b), the skin pieces of the corresponding batch were not used. For human skin, the skin integrity was assessed with trans-epidermal water loss (TEWL) values between 0.5 and 13 g m^{-2} h^{-1} as described in (Kluxen et al., 2022). TEWL was measured with a vapometer (Delfin Technologies, Finland).

Before applying the pesticide in the donor compartment, the skin or membranes were conditioned for 30 min with the receptor solution in the Franz cell. After conditioning, the receptor solution was replaced by a fresh solution. Different ethanol concentrations were tested in the receptor fluid, in the range from 0 to 50 % (v/v), and also 5 % BSA in saline (OECD, 2004, 2022).

At the donor compartment, chlorpyrifos (8.8 mg/mL) was applied in 25 μl acetone for a surface dose of 400 μg/cm^2 (1 μmol/cm^2), equal to the dose in studies with human volunteers (Griffin et al., 1999). Acetone is a solvent that evaporates fast, well-accepted to not alter the skin permeability and, so, a convenient vehicle for applying hydrophobic toxicants on skin (Hopf et al., 2018; Sartorelli et al., 1998). During the experiment, the Franz cells were kept at 32 °C and contained a magnetic stirrer rotating at 600 rpm in the receptor compartment. Samples (50 μl) were collected for the next 24h from the receptor at defined time points with immediate replacement of the collected volume. The concentration of chlorpyrifos was quantified, as described in 2.4, to obtain permeation curves. Flux was calculated by using at least 6 time points in the linear phase of permeation, and the lag time was computed from the interception of the linear permeation curve with the time axis. In the experiments using BSA in the receptor fluid, the pesticide was analyzed at 8 h after application in the donor compartment. For all the experimental conditions studied, triplicate assays were performed.

Additional assays were carried out to assess the potential adsorption of chlorpyrifos to the receptor compartment's glass when low-ethanol fluid was used, as described in Appendix B, and the results indicated very low adsorption (<10 %). The stability of chlorpyrifos in the receptor fluids was also evaluated and we found no significant differences in 1 mg/L solutions incubated at 32 °C for 24h (Fig. S1).

2.4. Chlorpyrifos quantification

The concentration of chlorpyrifos in the receptor compartment was quantified by reverse-phase HPLC, using an Agilent 1100 system equipped with a C18 column and UV detection at 225 nm (Marques-da-Silva et al., 2022). The sample injection volume was 20 μL, and the mobile phase consisted of acetonitrile:water (85:15) at a flux of 1 mL/min. For the quantification of the pesticide, considering the effect of the sample solvent in peak shape (Keunchkarian et al., 2006), calibration curves were obtained with standards dissolved in each of the different receptor compositions tested (examples in Fig. S2 of SI).

In the case of experiments using BSA in the receptor fluid, the samples passed by liquid-liquid extraction with hexane before HPLC. In the case of BSA, the pesticide in the 5 mL receptor volume at a concentration of 0.1 μg/mL was extracted twice for 30 sec with 0.5 mL of hexane, achieving an extraction yield of 87 ± 2 %. In these cases, the calibration curves were obtained with standards in hexane.

2.5. Determination of chlorpyrifos solubility

The solubility of chlorpyrifos in different solvents was determined by adding an excess amount of the pesticide to approximately 1 mL of each solvent, in triplicate. The mixtures were incubated at 32 °C, with agitation, for at least 48 h and not more than 72 h. The samples were centrifuged at 14 100 *g*, for 5 min, to separate the undissolved solid, and the supernatant was immediately collected. If necessary, a dilution step was performed in the same solvent where solubility was being tested. Then, the quantification was performed via HPLC as described in the previous section 2.4. To determine the chlorpyrifos solubility in 5 % BSA solution, the incubation and centrifugation were carried in the same way, and the hexane extraction as described in the previous section.

2.6. Measurement of contact angle

The contact angle related to the interaction of the different solvents with the different skin models was investigated for the external and internal faces of each model. The skin models were horizontally placed on the stage of an optical tensiometer Theta Lite (Biolin Scientific, Sweden) and at a lower level of the delivering drop syringe system. The moment the drop contacted the skin model, the capture of the contact angle was initiated using the software OneAttension for live analysis for 10 sec enclosing 100 analysis points. The analysis was performed using the Young-Laplace fit.

2.7. Upward migration of ethanol and chlorpyrifos from the receptor

To investigate ethanol permeation through the different barriers from the receptor compartment to the donor compartment, the Franz cell system was established as usual and ethanol was detected via

oxidation by acidified potassium dichromate. The assay setup was the same as described for the standard permeation assays in 2.3, with the skin models placed in the Franz cells at 32 °C (without conditioning). Once the appropriate fluid was completely added to the receptor compartment, a small plastic insert that fitted into the donor compartment was placed above the skin model surface, without contacting it, and 100 μl of the reactive solution that detects ethanol was added into the insert. In a few seconds, the donor was occluded so that the gaseous ethanol accumulated in the compartment and reacted with the solution in the insert. The solution was a 1:1 mixture of potassium dichromate (0.1N) and sulfuric acid (98 %), initially having an orange color that gradually fades with the ethanol reduction of dichromate (University of Canterbury, n.d.). A photograph of the system (insert in the donor compartment) was taken at time points 0, 3, 8, 15, and 30 min, and the decolorization of the dichromate solution monitored the accumulation of ethanol in the gaseous phase of the donor compartment. The dichromate decolorization was calculated using the Image J software for image processing and analysis by defining a uniform region of interest (ROI) for all the images analyzed. The mean values of red, green, and blue (RGB) intensities were obtained from the histogram of each ROI. The yellow intensity was obtained by adding the red and green intensities and, then, dividing by the blue intensity for normalization. The yellow intensity at time zero of the assay was considered 100 %, and the decolorization percentage was calculated by subtracting the yellow intensity at each subsequent time point.

The migration of chlorpyrifos through the different barriers, conditioned with the receptor fluids, was studied in upward permeation assays carried out with the pesticide loaded in the receptor compartment and measured in the upper surface of the skin model tested. The Franz cells were mounted and the initial 30-min conditioning of the skin or synthetic membranes was carried out as described in section 2.3. Then, chlorpyrifos was added to the receptor compartment at a concentration of 0.16 mg/L (10 times inferior to the solubility in saline solution). After 8h at 32 °C, 100 μl of pure ethanol was deposited over the donor facing surface of the skin model and, after 1 min, 50 μl of the extractant was collected and injected into the HPLC system for quantification as described in section 2.4.

3. Results and discussion

This study encloses experimental data of the permeation of chlorpyrifos through 4 different skin models: silicone and STRAT-M® membranes, and human and pig skin *ex vivo*. Previously studied with monkey and human skin *ex vivo* (Griffin et al., 2000; Moore et al., 2014; Sartorelli et al., 1998), the results obtained using receptors with ethanol (50 %) and BSA were significantly different (Table 1). Therefore, different receptor fluids were investigated in this work beyond the ones containing 5 % BSA or 50 % ethanol recommended by the guidelines (OECD, 2004, 2022).

In all the permeation assays, a chlorpyrifos dose of 400 μg/cm^2 (1 μmol/cm^2) was applied on the surface of the skin models studied, the same dose previously used in the human *in vivo* study of chlorpyrifos dermal absorption carried out by Griffin et al. (1999). This dose fits with the maximum value found in the skin of the thighs of cotton field workers (applicator) exposed to chlorpyrifos (Farahat et al., 2010). Moreover, as deduced in the SI (Appendix A), this dose saturates the skin stratum corneum so that maximum fluxes can be obtained to allow for direct comparison of the *in vitro* permeation fluxes and the *in vivo* absorption rate presented in Table 1.

3.1. Comparison of standardized assay receptor fluids containing ethanol and serum albumin

Facing the substantial differences between the published *in vitro* skin permeation data using ethanol- and BSA-containing receptors, and also relative to the *in vivo* data (Table 1), we started by carrying assays of chlorpyrifos permeation in the standardized conditions. Following the OECD guidelines, dermal permeation was investigated with the recommended 5 % (w/v) BSA or 50 % (v/v) ethanol in the receptor fluid (OECD, 2004, 2022). The 4 different skin models were tested and an 8h permeation time was adopted, a period that simulates occupational exposure during a work shift and also followed in the human *in vivo* study of dermal exposure by Griffin et al. (1999).

Table 2 shows the results from the determination of the pesticide in the receptor fluids. Our results with human skin were close to those obtained by other authors in similar conditions and confirmed that the 50 % ethanol receptor favors chlorpyrifos permeation. The amount of chlorpyrifos found in 50 % ethanol, after 8 h permeation, was 100 times superior to the measured in BSA-containing receptors (Tables 1 and 2). Regarding the results obtained with pig skin and the STRAT-M® membrane, they were not significantly different from the observed with human skin, both using the BSA and the ethanolic receptor fluids (Table 2). Dissimilarly, the assays performed with the silicone membrane presented higher values for the concentration of chlorpyrifos in the receptor fluid after 8h when compared to the human skin *ex vivo* (Table 2).

It should be noted that the permeation of chlorpyrifos to the two receptor fluids was not limited by the solubility of the pesticide in any of the cases. The solubility in the BSA and in the ethanolic solution was 208 and 1043 mg/L (Table S3), much higher than the concentrations attained in the corresponding assays (Table 2). The solubility measured in BSA is very close to the value calculated considering the high affinity

Table 2
– Permeation of chlorpyrifos through different skin models, in 8h, using receptor fluids containing bovine serum albumin (BSA) or ethanol in the diffusion cells. The dose of chlorpyrifos applied was 400 μg/cm^2. The quantity of permeated chlorpyrifos was normalized to the permeation area of the corresponding diffusion cells. The results from the present work are shown as means ± SE from at least triplicate assays for each condition.

Skin Model	BSA in the receptor fluid		Ethanol (50 % v/v) in the receptor fluid		References
	Chlorpyrifos concentration (μg/mL)	Chlorpyrifos permeated *per* area (μg/cm^2)	Chlorpyrifos concentration (μg/mL)	Chlorpyrifos permeated *per* area (μg/cm^2)	
Human skin	0.080[a]	0.017[a]	–	–	Moore et al. (2014)
Human skin	–	–	3.9	11.7	Griffin et al. (2000)
Human skin	0.034 ± 0.008[b]	0.27 ± 0.07[b]	5.4 ± 2.8	42.6 ± 21.9	Our work
Pig skin	0.034 ± 0.014[b]	0.27 ± 0.11[b]	7.7 ± 4.0	60.2 ± 31.3	Our work
Silicone membrane	1.77 ± 0.34[b]	13.8 ± 2.7[b]	22.5 ± 0.9	175.8 ± 7.0	Our work
STRAT-M® membrane	0.023 ± 0.003[b]	0.18 ± 0.02[b]	2.6 ± 0.5	20.3 ± 3.9	Our work

[a] 2 %(w/v) BSA in the receptor fluid.
[b] 5 %(w/v) BSA in the receptor fluid.

(logK>5) and one binding site for chlorpyrifos in BSA (Coelho et al., 2025), 264 mg/L (Table S3).

When comparing to the *in vivo* absorption data, namely with the amount of chlorpyrifos calculated to be dermally absorbed (Table 1), the use of ethanol-containing receptors in the *in vitro* assay afforded a better estimation than the BSA receptors. During 8 h skin exposure to chlorpyrifos, receptors with concentrations of BSA up to 5 % enabled the permeation of less than 0.3 μg/cm^2, clearly below the 3.64 μg/cm^2 calculated from *in vivo* data (Table 1). Additionally, when BSA is used in the receptor solution a Tlag of 6h was reported (Moore et al., 2014), while no lag period was observed in assays using 50 % ethanol in the receptor (Griffin et al., 2000), which is more coherent with the rapid absorption of chlorpyrifos observed in the human *in vivo* studies (Griffin et al., 1999) with a Tlag inferior to 1h (Table 1).

The Tlag and the amount of chlorpyrifos permeated to receptors with 50 % ethanol (Table 1) are in general more compatible with the *in vivo* data. However, doubts on the validity of these estimates also arise considering their high values and the absence of a justification to use ethanol at 50 % in the receptor. As presented in the Introduction, the use of high percentage ethanolic receptors has been questioned and requires additional support. So, we decided to investigate more thoroughly how the concentration of ethanol in the receptor fluid affects the permeation of chlorpyrifos *in vitro*.

3.2. *Effect of the ethanol concentration in the receptor fluid on the permeation of chlorpyrifos through skin and synthetic membranes*

Aiming to understand how ethanol influences the permeation of chlorpyrifos, different concentrations of ethanol in the receptor fluid were tested with each skin model. The kinetic profiles obtained are presented in Fig. 1 and the calculated permeation parameters are in Table 3. The concentrations of 0 (saline solution), 10, 30, and 50 % of ethanol were studied in general, except with the STRAT-M® membrane, with which no permeation was detected with 10 % ethanol and lower concentrations were not assessed (Fig. 1D). This is in line with previous work pointing that the lipophilic compounds tend to have lower permeation through STRAT-M® membrane to a saline (no ethanol) receptor (Haq et al., 2018).

The results show a shift of the permeation profiles when changing the percentage of ethanol in the receptor fluid. The higher percentages of ethanol in the receptor fluid caused a faster permeation of chlorpyrifos and this effect was evident across all the models tested (Fig. 1).

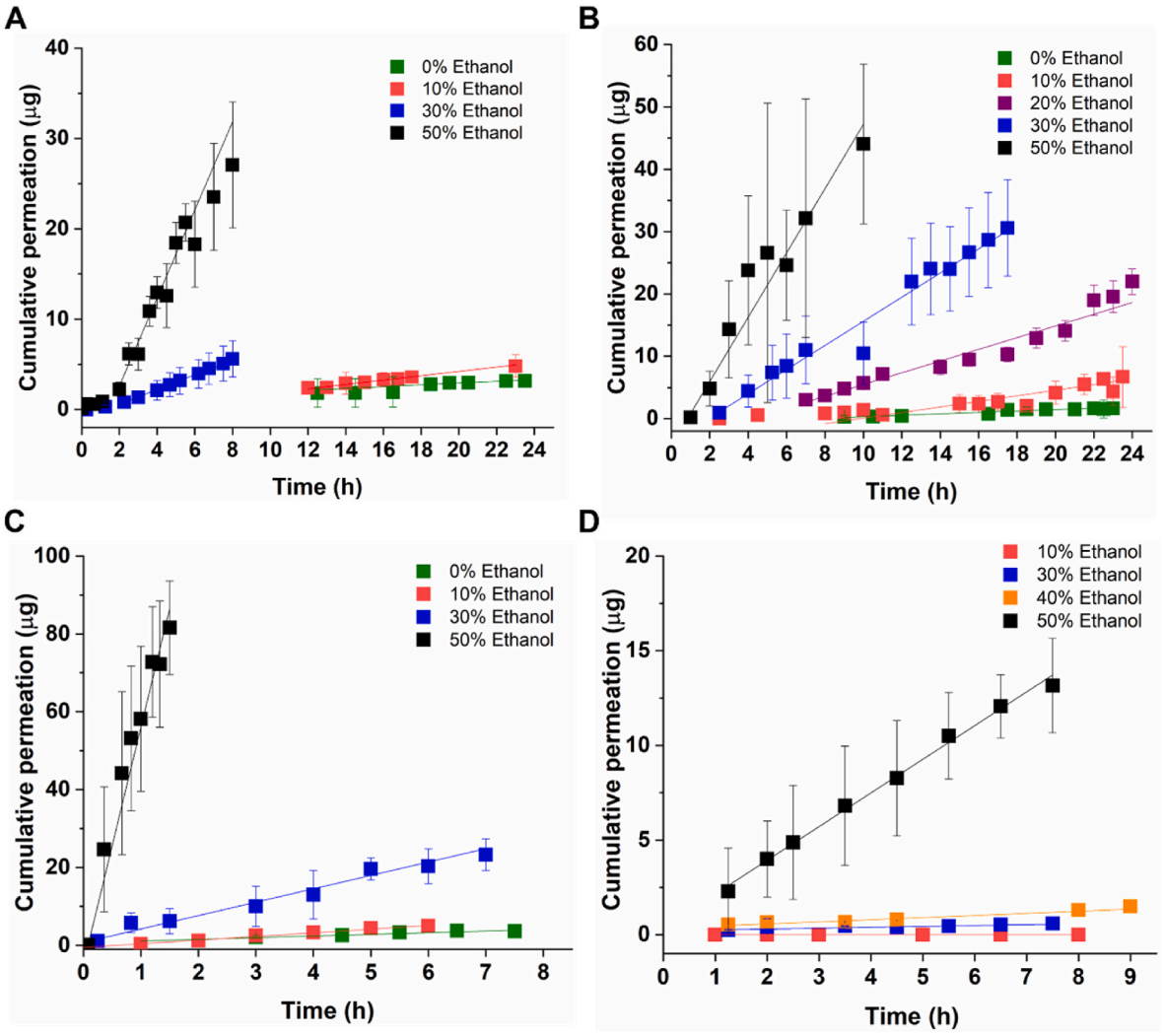

Fig. 1. – Cumulative permeation curves of chlorpyrifos through human skin (A), pig skin (B), silicone membrane (C), and STRAT-M® **membrane (D), using different concentrations of ethanol in the receptor fluid of the diffusion cell.** The dose of chlorpyrifos applied was 400 μg/cm^2. The permeation assays were performed at 32 °C in static Franz cells with a permeation area of 0.64 cm^2. The lines are the best linear regression fits of the experimental data used to calculate fluxes, and the lowest correlation coefficients (R^2) observed were 0.9, 0.8, 0.9, and 0.8 for human skin, pig skin, silicone, and STRAT-M® membrane, respectively. All the experimental results shown are means ± SE from at least triplicate assays for each condition.

Table 3
– Permeation of chlorpyrifos through different skin models and using varying concentrations of ethanol in the receptor fluid of the diffusion cell. The dose of chlorpyrifos applied was 400 μg/cm^2. The permeation assays were performed at 32 °C in static Franz cells with a receptor volume of 5 mL and a permeation area of 0.64 cm^2. All the results shown are means ± SE from at least triplicate assays for each condition.

Skin Models	Ethanol in the receptor fluid (%)	Permeation flux (μg cm^{-2}h^{-1})	Lag time (h)	Concentration of chlorpyrifos permeated at 8h (μg/mL)	Quantity of chlorpyrifos permeated at 8h (μg)
Human Skin	50	6.4 ± 2.8	1.1 ± 0.2	5.4 ± 2.8	27.1 ± 13.9
	30	1.31 ± 0.36	1.3 ± 0.4	1.1 ± 0.4	5.6 ± 2.0
	10	0.36 ± 0.04	1.5 ± 1.5	0.3 ± 0.1	1.5 ± 0.2
	0	0.23 ± 0.03	1.2 ± 0.8	0.2 ± 0.1	1.0 ± 0.5
Pig Skin	50	12.5 ± 4.2	1.55 ± 0.13	7.7 ± 4.0	38.4 ± 20.1
	30	3.5 ± 0.6	4.2 ± 1.5	2.7 ± 2.3	13.5 ± 11.5
	20	1.8 ± 0.2	5.5 ± 0.5	1.04 ± 0.53	5.3 ± 2.8
	10	0.73 ± 0.09	7.5 ± 1.6	0.17 ± 0.25	0.7 ± 1.3
	0	0.28 ± 0.03	8.3 ± 1.8	0 ± 0	0 ± 0
Silicone membrane	50	90.3 ± 11.6	0.05 ± 0.02	22.5 ± 0.9	112.3 ± 4.3
	30	4.8 ± 0.1	0.3 ± 0.2	4.9 ± 1.4	24.3 ± 6.8
	10	1.5 ± 0.1	0.6 ± 0.3	1.4 ± 0.2	6.8 ± 0.8
	0	0.9 ± 0.1	0.2 ± 0.2	0.8 ± 0.1	3.8 ± 0.4
STRAT-M® membrane	50	2.8 ± 0.1	0.7 ± 0.5	2.6 ± 0.5	13.0 ± 2.5
	40	0.116 ± 0.021	0 ± 0	0.26 ± 0.05	1.3 ± 0.2
	30	0.085 ± 0.011	0 ± 0	0.12 ± 0.03	0.6 ± 0.1
	10	0 ± 0	–	(<detection limit)	(<detection limit)

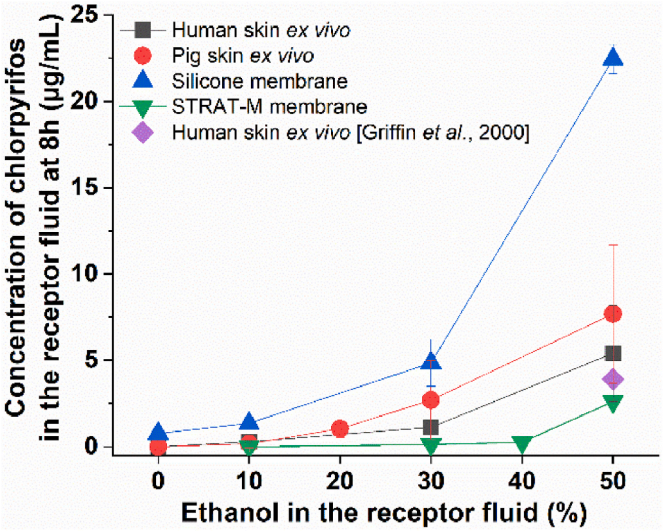

Fig. 2. – Chlorpyrifos that permeated through different skin models, in 8h, using receptor fluids containing different concentrations of ethanol in the diffusion cell. The purple lozenge represents the data obtained by Griffin et al. (2000) using human skin *ex vivo*. The permeation assays were performed at 32 °C in static Franz cells with a receptor volume of 5 mL and a permeation area of 0.64 cm^2. All the results shown are means ± SE from at least triplicate assays for each condition.

Consistently, a higher concentration of 8-h permeated pesticide was measured with higher percentages of ethanol in the receptor fluid (Fig. 2 and Table 3). With pig skin and silicone membrane, the effect of ethanol could be noticed with concentrations inferior to 30 %, but higher ethanol concentrations also reflected in the 8h-permeated chlorpyrifos with human skin and the STRAT-M® membrane.

These results are in agreement with another study that also reported a higher permeation of lipophilic organophosphorus compounds through human epidermis when assayed with increasing proportions of ethanol in the receptor (Thors et al., 2016). In a different work, ibuprofen applied in ethanol-water vehicles permeated silicone and human skin faster when using higher ethanol percentages in the vehicle (Watkinson et al., 2009).

A more refined analysis of the effect of ethanol can be achieved by considering the parameters of chlorpyrifos permeation - flux and Tlag - calculated from the kinetic curves (Fig. 1). The flux is the mass of the compound passing through the skin area *per* time unit (μg cm^{-2} h^{-1}). And, Tlag is the interception of the linear part of the permeation curve with the time axis (Hopf et al., 2020; OECD, 2022). The flux and Tlag of chlorpyrifos permeation using the different skin models and receptor conditions are presented in Table 3 and Fig. 3. Moreover, additional values obtained in other works with *ex vivo* and *in vivo* human skin (from Table 1) are also represented for comparison in Fig. 3A.

As expected, the percutaneous fluxes of the lipophilic pesticide to saline receptor (0 % ethanol) were very low (Table 3). Remarkably, the human and pig skin values between 0.2 and 0.3 μg cm^{-2} h^{-1} were in good agreement to the prediction in Appendix A (SI) based on general principles of dermal permeation (Mitragotri et al., 2011; Silva et al., 2021a) and partition coefficients estimated for chlorpyrifos (Raykar et al., 1988; Van Der Merwe and Riviere, 2005). However, as Fig. 3A illustrates, the ethanol concentration in the receptor fluid increases the permeation flux of chlorpyrifos in a skin model-dependent way. The crossing through pig skin and the silicone membrane is more sensitive to ethanol, while human skin and the STRAT-M® membrane show minor and robust responses only to the 50 % concentration. The Tlag of chlorpyrifos detection in the receptor compartment after penetration through pig skin is also strongly influenced by the ethanol in the receptor fluid (Fig. 3B). With saline (0 % ethanol) in the receptor compartment, the Tlag is 8.3 h, but it gradually decreases to less than 2h by increasing ethanol to 50 % (Table 3). The Tlag with the other skin models was very short (<2h), even for saline receptor, hence an eventual decrease caused by ethanol would be difficult to establish (Fig. 3B).

Worth noting, the permeation flux and Tlag herein obtained with human skin and 50 % ethanol are close to the results reported by Griffin et al. (2000) - represented by the lozenges in Fig. 3. The minor differences in the values can be due to variability in the skin and other experimental conditions like the applied dose of chlorpyrifos (Tables 1 and 3). Very important, the human skin *ex vivo* and the STRAT-M® membrane returned flux and Tlag values close to the human dermal absorption data estimated in the Griffin et al. (1999) *in vivo* study (Table 1). No additional studies were found in the literature on chlorpyrifos permeation with other ethanol concentrations in the receptor compartment, nor using pig skin or synthetic membranes.

The pig skin and the silicone membrane assayed with 50 % ethanol,

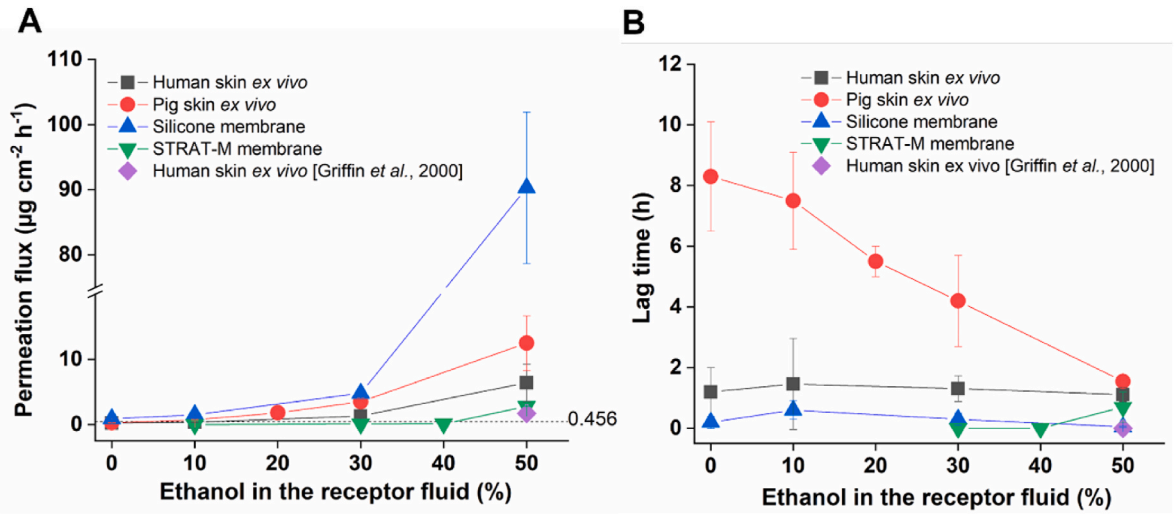

Fig. 3. – Flux (*A*) and lag time (B) of chlorpyrifos permeation through different skin models and using varying concentrations of ethanol in the receptor fluid of the diffusion cell. The purple lozenge represents the data obtained by Griffin et al. (2000) using human skin *ex vivo* (Griffin et al., 2000), and the horizontal dashed line in panel A marks the *in vivo* human absorption rate reported by Griffin et al. (1999). The results shown were calculated from the kinetic data in Fig. 1 and are means ± SE from at least triplicate assays for each condition.

afforded fluxes of chlorpyrifos clearly superior to the estimated *in vivo* dermal absorption rate, 0.456 µg cm^{-2} h^{-1} - depicted by the dashed line in Fig. 3A. Nevertheless, both the synthetic membranes and the human skin, when assayed with lower concentrations of ethanol (10, 30 or 40 %), returned flux and Tlag values equally compatible with the *in vivo* estimates (Fig. 3A and B).

These synthetic membranes have been studied as alternative models of skin, but only tested with aqueous receptor fluids. Uchida et al. (2016) proposed the silicone membrane as a validated model of human skin permeation for different compounds (Uchida et al., 2016), and our previous study with caffeine was in coherence (Silva et al., 2021b). In the case of STRAT-M®, according to the supplier, it is designed for screening different compounds, including pesticides (Merck, 2018). This membrane is seen as an alternative for the evaluation of drug transdermal diffusion (Simon et al., 2016) and it was previously described as an alternative membrane in skin permeation studies (Haq and Michniak-Kohn, 2018; Uchida et al., 2015) for different compounds. Nevertheless, although different lipophilic compounds were tested in the study of Uchida and colleagues (2015), the maximum octanol-water partition coefficient (log $K_{o/w}$) tested was 3.5. Data for more lipophilic compounds can be found in Merck (2018) brochure, but the composition of the receptor fluid used in the assays is not indicated. In the present study, STRAT-M® was tested for chlorpyrifos which presents a logKow of 4.96 and for the first time with a range of receptors with ethanol up to 50 % as recommended by the guidelines.

From our results, the Tlag obtained with the synthetic membranes are inferior to 1h for all the ethanol concentrations in the receptor fluid tested in this work (Fig. 3B), similar to the observed on human skin *in vivo* absorption (Griffin et al., 1999). Although, when analyzing the fluxes obtained, the STRAT-M® membrane is the one that approximates from the fluxes obtained in the *in vivo* study (Fig. 3A) supporting it as a good synthetic membrane to be used as alternative models in risk assessment procedures for chlorpyrifos.

The fact that synthetic membranes and human skin returned valid data with receptor fluids containing less than 50 % ethanol suggests that the percentage of ethanol in the *in vitro* permeation assay can be reduced. This is especially relevant considering some results point that ethanol is affecting the permeation process in other way(s) beyond the chlorpyrifos solubility increase, as discussed in the following sections.

3.3. Solubility of chlorpyrifos in the receptor fluids

The reason for using BSA or ethanol in the assay of lipophilic compounds is to ensure their solubility in the receptor fluid so that partition to the receptor does not act as a barrier to the permeation. According to the recent OECD 2022 guideline (OECD, 2022), the solubility of the test compound should be at least 10 times higher than the maximum concentration reached in the receptor fluid at the end of the permeation assay.

The water solubility of chlorpyrifos is estimated as 4.8 mg/L (Silva et al., 2021a) and values in this order were measured in the receptor fluids of the permeation assays (Tables 2 and 3), suggesting that indeed the solubility of the pesticide might not be sufficient in some conditions.

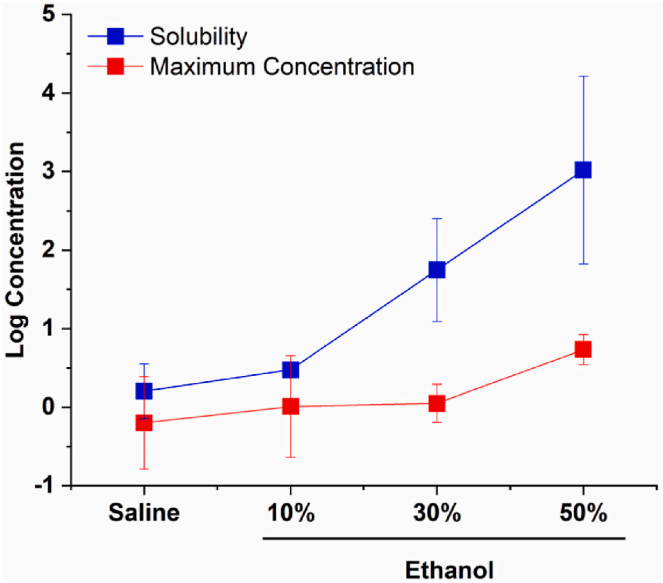

Fig. 4. – Chlorpyrifos solubility in the different solvents used as receptor fluids in permeation assays and maximum concentrations reached in the respective assays of permeation through human skin. The maximum concentrations reached were obtained from the kinetic data in Fig. 1A. Logarithm of concentrations in mg/L. The results shown are means ± SE from triplicate measurements with each solvent.

We decided to determine the solubility of chlorpyrifos in the different receptor fluids and obtained the values presented in Table S3 and plotted in Fig. 4. The results showed that ethanol causes a great increase in the solubility of chlorpyrifos, almost three orders of magnitude (Fig. 4), from 1.6 mg/L in saline (0 % ethanol) to 1.0 g/L in 50 % ethanol (Table S3). The solubility of chlorpyrifos in the ethanolic fluids was also estimated by the log-linear model (Li and Yalkowsky, 1994) and the values obtained are similar to those experimentally determined (Table S3).

Collecting the higher concentrations reached in the permeation assays with human skin (data from Fig. 2 and Table 3), it could be observed that the chlorpyrifos concentrations accumulated in the receptors with saline and 10 % (v/v) ethanol were close to the corresponding solubilities (Fig. 4). The maximum concentrations were 0.6 and 0.9 mg/L, respectively. In contrast, the maximums obtained with 30 and 50 % (v/v) were 1.1 and 5.4 mg/L, respectively. These values are more than 10 times inferior to the solubilities, ensuring that the solubility of the test compound in the receptor fluid is not rate limiting of the permeation process. Moreover, the difference between the receptor concentrations and the solubilities is ample with 30 % and 50 % (v/v) ethanol (Fig. 4), as well as with 5 % BSA (w/v), indicating that sink conditions are maintained through the 8-h permeation assays (Table 2 and Fig. 2), so some other factor must be responsible for the higher fluxes observed with 50 % ethanolic receptor (Fig. 3A).

The same can be concluded regarding the assays with other skin models. The concentrations reached in the receptors of pig skin and STRAT-M® assays are similar or lower than those of human skin, and even those observed with the more permeable silicone membrane are also far from saturating the receptors containing 30 and 50 % ethanol or 5 % BSA. The robust acceleration of chlorpyrifos flux through pig skin and silicone to 50 % ethanol (Fig. 3A) indicates that ethanol favors some other step of the permeation process. In the same direction, a study with a less lipophilic compound - thymoquinone (log $K_{o/w}$ 2.2) - showed that the flux of skin permeation could increase almost ten times with the presence of ethanol in the receptor fluid, although the solubility increase obtained with ethanol addition (1:1) was modest (from 0.4 to 0.8 mg/mL), suggesting that the faster permeation is not simply caused by the solubility increase (Haq and Michniak-Kohn, 2018).

This is reinforced by the ethanol-induced decrease in Tlag of chlorpyrifos permeation so evident with pig skin (Fig. 3B). The Tlag of permeation is not expected to depend on the solubility of the permeant in the receptor fluid. Assuming a simple Fickian diffusion, Tlag is directly proportional to the square of the thickness of the barrier and inversely proportional to the diffusivity of the permeant within the barrier (Hopf et al., 2020). Pig skin is substantially thicker than the other skin models studied (Table S2), which contributes to the larger Tlag (Fig. 3B), but the decrease observed with ethanolic receptors indicates that the properties of the barrier are altered on interacting with ethanol.

3.4. Contact angle of the receptor fluids

In addition to solubility, the adherence of the receptor fluids to the permeation barriers might also affect the kinetics of mass transfer between the two phases. This step is usually not considered limiting of the permeation process with skin and aqueous base receptors, but quite distinct barriers and receptor fluids are under comparison here. In a work with haloperidol, fluids with higher skin wettability - measured by the contact angle - promoted the interaction of the drug with the skin surface and resulted in higher permeation (Azarbayjani et al., 2010). Therefore, we measured the contact angles of the saline and ethanolic solutions in the external and internal surfaces of the different barriers. The values obtained are in Table S4 and represented in Fig. 5. We have also measured the contact angle of the BSA solution and of human plasma as a fluid mimicking the tissues' intercellular fluid (Table S4).

The plasma, BSA and saline solutions produced similar results with each surface and barrier (Table S4). In the external surface of human skin and STRAT-M® membrane, these fluids had contact angles around 65–70°, while in pig skin the results were significantly lower and in the silicone membrane it reached 100°. Silicones in general form hydrophobic films with low surface tension (Colas and Curtis, 2013). The angles obtained for these fluids in the external face of the human skin are comparable to those obtained with water in other works (Azarbayjani et al., 2010; Kovalev et al., 2014). In the internal surface of human and pig skin, low contact angles were obtained with the 3 fluids – inferior to 20° in most cases (Table S4). In contrast, the contact angles with STRAT-M® and silicone were higher than 100°, indicating a strong hydrophobicity of the internal surfaces of these synthetic membranes.

When ethanol was present in the fluid, the contact angles decreased in a concentration-dependent manner, except if the angle was already very small (Fig. 5). In the external surface, significant differences between the barriers persisted through the whole interval of ethanol concentrations. The contact angles followed the order: pig skin < STRAT-M® = human skin < silicone membrane (Fig. 5A). In this context, it can be noted that when the acetone vehicle was applied on the top of skin models at the beginning of the permeation assays, no significant differences were observed in the spreading of the solvent

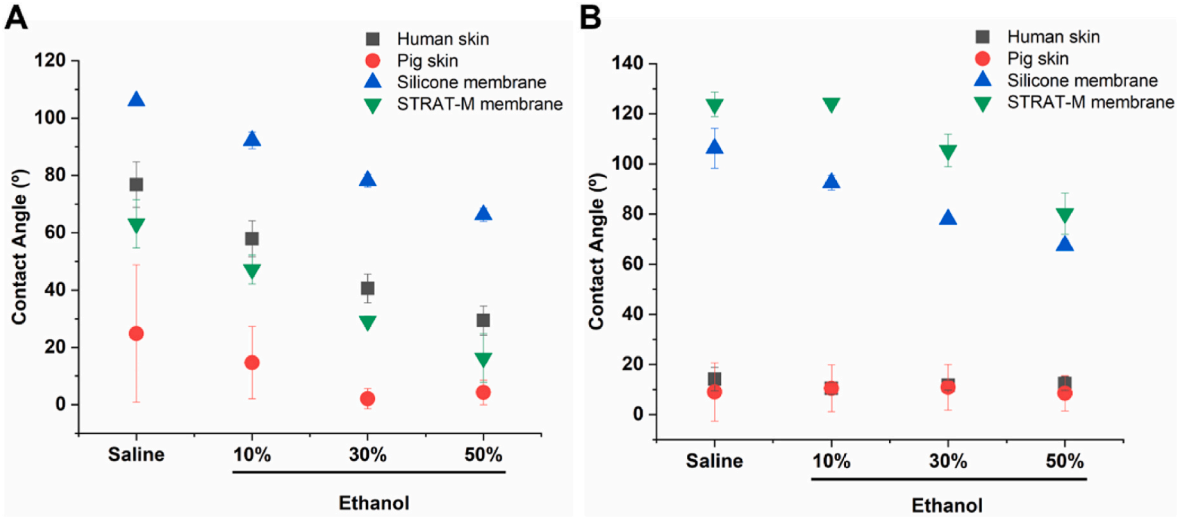

Fig. 5. – Contact angle of skin and synthetic membranes, external (A) and internal (B) surfaces, with the receptor fluids containing varying concentrations of ethanol. The results shown are means ± SE from at least triplicate measurements for each condition.

through the surface of the barriers in contact with different receptor fluid compositions, nor in the evaporation that occurred in a few minutes in all cases.

As for the contact angles obtained for the internal surface, the decreasing trend with the percentage of ethanol in the drop was also observed with the synthetic membranes (Fig. 5B). In human and pig skin, ethanolic fluids also showed low contact angles and insensitive to the concentration of ethanol, confirming an excellent wettability by all the fluids studied as receptors (Fig. 5B and Table S4). Thus, the faster permeation of chlorpyrifos through skin to high percentage ethanolic receptors (Fig. 3B) cannot be justified by a tighter adherence between the barrier and the fluid. In the same way, very different fluxes were observed through the silicone and STRAT-M® membranes with receptor fluids that show similar contact angles with these membranes, namely the 50 % ethanol (Fig. 3A and 5B).

The decrease in the contact angle of the ethanolic fluids, up to 50 % (v/v) ethanol, with the synthetic membranes indicates a closer adherence favorable to the fast partition of solutes from these barriers to the receptor fluids during permeation. However, the contact angles of these fluids with human and pig skin (internal surfaces) are much smaller, so more favorable, and show no variation with the percentage of ethanol (Fig. 5B). Despite that, the permeation fluxes with the skin types are intermediate of the synthetic membranes and all increase with 50 % ethanolic receptor (Fig. 3A). Therefore, the overall data indicates that the solute transfer from the barriers under study to the receptor fluids is not a rate-limiting step.

3.5. Crossing of ethanol through the barriers to the donor compartment and enhancement of chlorpyrifos permeability

Keeping in mind that using a 50 % ethanol receptor is recommended in the guidelines to assess the dermal absorption of hydrophobic compounds, we decided to investigate further the potential factors justifying the observed increase in chlorpyrifos flux with high percentage ethanol receptor fluids (Fig. 3A). From the previous results, the faster permeation flux with 30 and 50 % ethanol receptors could not be supported by the higher solubility of chlorpyrifos nor by a favored adherence of the fluids to the skin models. However, ethanol is known to partition into the skin and function as a permeation enhancer in different ways (Berner et al., 1989; Gupta et al., 2020; Watkinson et al., 2009).

We hypothesized that ethanol from the receptor compartment penetrates the permeation barriers and enhances their permeability. Investigating these hypotheses experimentally required adaptations of the conventional setup of the permeation assay, as detailed in the Methods section 2.7, to study the penetration of ethanol and chlorpyrifos in controlled conditions.

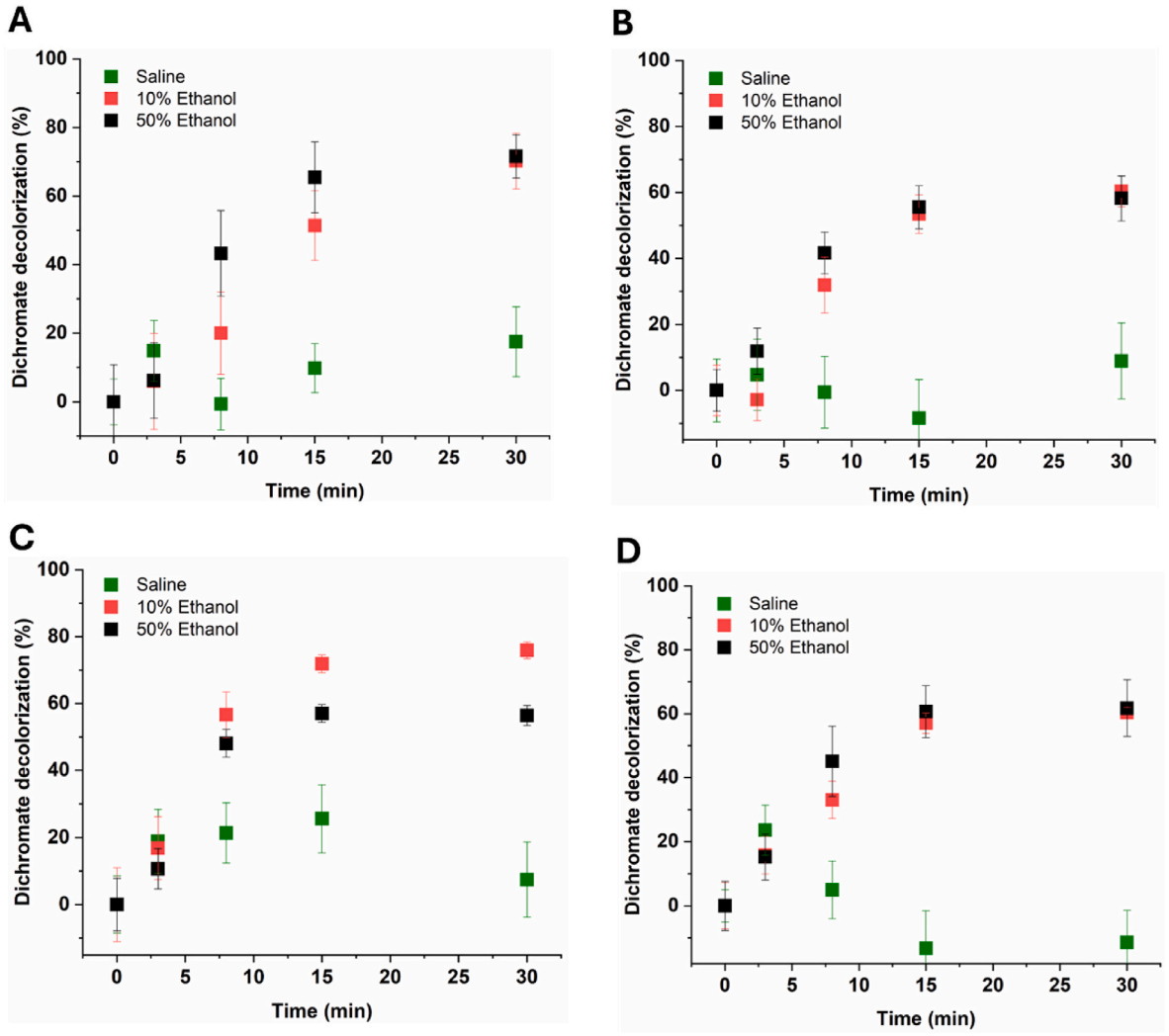

Fig. 6. – Kinetics of ethanol accumulation in the donor compartment of diffusion cells by crossing human skin (A), pig skin (B), silicone (C), and STRAT-M® **(D) from the receptor compartment containing different fluid compositions.** The dichromate decolorization by ethanol was calculated relative to the initial orange color of the acidified potassium dichromate solution placed in the donor compartment of each assay.

3.5.1. Detection of ethanol in the donor compartment

The crossing of ethanol from the receptor fluid through the barriers was possible to monitor by the decolorization of a dichromate solution in the donor compartment of the Franz cell. Fig. S3 in SI shows photographs of the dichromate solutions during the conditioning of the different skin models with receptors of saline, 10 and 50 % ethanol. The gradual decolorization of the initial orange dichromate solution was evident when ethanol was present in the receptor fluid (Fig. S3), and it is explained by the reduction of dichromate (University of Canterbury, n. d.) by the ethanol reaching the donor compartment of the Franz cells. When the receptor contained the saline solution, no significant changes were observed in the color of the dichromate solution, even at the end of the 30-min incubation (Fig. S3).

The kinetics of the decolorization was quantified by image analysis and the results in Fig. 6 show that ethanol penetrates and crosses the four skin models to the donor compartment in few minutes. This adds to a previous observation that ethanol permeates the skin after application on the external surface (Berner et al., 1989). Important to note, the silicone membrane was the most permeable to ethanol, as 50 % decolorizations were achieved in less than 10 min (Fig. 6C)

In the standard permeation assay, the skin and membranes are conditioned for 30 min with the receptor fluid before the test compound is applied in the donor compartment. The present results demonstrate that a few minutes are sufficient for ethanol to diffuse inside the barriers and even volatilize at the donor-facing surface. This can highly affect the permeation of chlorpyrifos as ethanol increases the skin permeability (Gupta et al., 2020).

3.5.2. Effect of the barrier conditioning fluid on the chlorpyrifos permeability

The presence of ethanol in the skin models studied is plausible to influence the overall permeability of hydrophobic compounds, such as chlorpyrifos, by increasing the partition/solubility of the compounds in the stratum corneum and by enhancing their diffusivity through the skin (Gupta et al., 2020; Haq and Michniak-Kohn, 2018; Pershing et al., 1990). Both effects can be expected to depend on the concentration of ethanol in the receptor fluid conditioning the permeation barrier.

The driving force for skin permeation is expressed by the difference in concentration of the compound between the stratum corneum and the receptor (Lian et al., 2008; Mitragotri et al., 2011; Pershing et al., 1990). Therefore, the conventional permeation assay with the tested compound applied in the donor compartment was not suitable to investigate the effect of ethanol presence within the skin on the chlorpyrifos diffusivity. If the solubility of the compound in the stratum corneum increases with ethanol, the driving force for permeation also increases, so the effect on the diffusivity could not be isolated from the effect on the stratum corneum concentration. A different (inverted) permeation experiment was designed enabling to fix the same concentration gradient across the barriers, independently of the receptor fluid assayed. As specified in Methods section 2.7, the permeation barriers were mounted in the Franz cells and conditioned with the receptor fluids as in the standardized assay, and then a small volume of chlorpyrifos was added to the "receptor" compartment. A fixed amount of chlorpyrifos was added to reach a low concentration, well below the saturation of any of the receptor fluids studied. Afterwards, the upward permeation of chlorpyrifos was quantified by extracting the compound reaching the external surface of the skin and synthetic membranes in 8 h.

The HPLC quantification revealed that the chlorpyrifos found at the donor-facing surface of the barriers was higher when a higher percentage of ethanol was used in the receptor compartment (Fig. 7). This ethanol-dependent increase in superficial chlorpyrifos was observed with all the skin models studied, but the massive increase with the silicone membrane exposed to 50 % ethanol reinforces the sensitivity of this barrier to the high percentage ethanolic receptor fluid. Worth recalling, the greater increase in chlorpyrifos flux promoted by 50 % ethanol receptors in the standard permeation assay was also observed

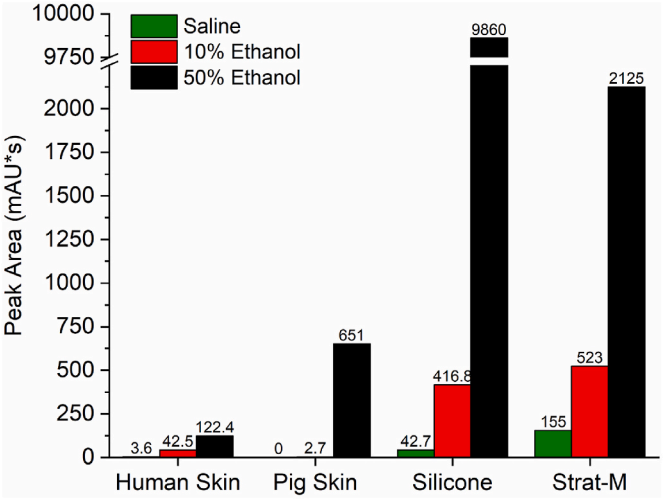

Fig. 7. – Amount of chlorpyrifos in the skin models' surface facing the donor compartment after upward migration from the receptor compartment containing different fluids. The skin and synthetic membranes were assayed with saline solution, 10 and 50 % ethanol in the receptor compartment. The initial concentration of chlorpyrifos in the receptor fluids was fixed (0.16 mg/L). The permeation occurred for 8 h, at 32 °C, and the superficial chlorpyrifos was measured by HPLC.

with silicone (Fig. 3A). Silicone materials can exhibit high permeability to gases and solutes due to low chain-to-chain interactions and high free volume (Colas and Curtis, 2013; Watkinson et al., 2009), which is plausible to facilitate the penetration of ethanol and the diffusion of chlorpyrifos.

The data obtained with the method of upward permeation under an identical concentration gradient between the two sides of the barriers demonstrate that ethanol from the receptor compartment of the Franz cells affects the permeation process by ways beyond the desired solubility increase. In the case of the skin, ethanol was described to penetrate lipid layers, disturb and extract lipids in a concentration-dependent manner, and create channels for molecules' passage (Bommannan et al., 1991; Gupta et al., 2020; Hatta et al., 2017). The properties of silicone are also known to be affected by ethanol (Tan et al., 2020), but we found no other studies of STRAT-M® with ethanolic solvents. Clearly, the use of ethanol in the receptor fluid of the dermal permeation assays needs more careful consideration, and additional studies of synthetic membranes like STRAT-M® in ethanolic fluids are needed to promote wider adoption of these skin surrogates in the assays.

4. Conclusions

In this study, experimental data on the permeation of chlorpyrifos through pig skin *ex vivo*, silicone and STRAT-M® membranes is provided for the first time, and in conditions directly comparable to the permeation through human skin *ex vivo* and the published *in vivo* absorption data. The *in vivo* data of Griffin and colleagues (1999) should be regarded with caution as the absorption rate of chlorpyrifos was simply computed from the total amount of metabolites collected in urine divided by exposure time (Table S1). In spite of the limitation of this approach, it provides the best available estimative of the dermal absorption rate of chlorpyrifos.

The use of receptor fluids containing BSA and ethanol in the *in vitro* permeation assay was investigated diligently. None of the guidelines-recommended 5 % BSA and 50 % ethanol receptors in Franz cells returned completely acceptable results. With the BSA receptor, and despite the notable increase in solubility of chlorpyrifos in this fluid, the *in vitro* permeation through skin and the STRAT-M® membrane was well below the reported *in vivo* human data (Griffin et al., 1999). In this

concern, skin permeability results compiled in the recent SkinPiX dataset also indicate that the presence of BSA (2 %) in receptor fluids has no major influence in the measured transcutaneous permeation, at least of compounds having log $K_{o/w}$ up to 1.6 (Chedik et al., 2024). Nevertheless, when we used the 50 % ethanol receptor, some of the permeation results were quite high - including with human and pig skin - reinforcing previous indications that high percentage ethanolic receptors can overestimate the human skin permeability (Silva et al., 2021a; Thors et al., 2016).

The influence of different concentrations of ethanol in the receptor fluid on the permeation of the hydrophobic pesticide through skin and the synthetic membranes is another contribution for improving the standardization of the *in vitro* dermal absorption assay and the wider adoption of non-animal skin models. The silicone membrane and the pig skin were more sensitive to ethanol than human skin and STRAT-M®. The permeation data herein obtained allow us to propose STRAT-M® as a useful non-animal model to investigate the percutaneous permeation of chlorpyrifos, simulating human skin exposure to the pesticide, even when using ethanolic receptors needed to assay lipophilic compounds. Nevertheless, human skin explants remain essential to integrate the effects of formulation ingredients, different permeation pathways and metabolism on the *in vitro* dermal absorption of compounds (Mitragotri et al., 2011; Olkowska and Gržinić, 2022).

Ethanol-containing receptors in the *in vitro* assay afforded a better estimate of chlorpyrifos dermal absorption than the BSA receptors. However, the increase of ethanol in the receptor fluid led to a faster permeation of chlorpyrifos through every skin model tested, especially with the higher 50 % concentration tested, that neither solubility nor barrier-receptor fluid adherence could justify. On the contrary, with all skin models we found that ethanol migrates from the receptor to the donor compartment in a concentration-dependent way, and the ethanol within the permeation barriers increases chlorpyrifos diffusivity. Therefore, it becomes very valuable that receptor fluids with for example 30 % ethanol also ensure sufficient chlorpyrifos solubility for assays longer than 8 h, and yielded permeation parameters – at least with human skin and synthetic membranes – that are predictive of human *in vivo* dermal absorption.

Future studies utilizing spectroscopic (Bommannan et al., 1991; Hatta et al., 2017; Kwak et al., 2012) and histological techniques can be valuable for refining the effects, at the molecular and structural levels, of different concentrations of receptor's ethanol on the skin during dermal permeation experiments. Also important for future research, there is a need for information on the integrity of synthetic membranes in high ethanolic fluids.

Finally, the results presented in this study support the following recommendations.

- The percentage of ethanol in the receptor fluid of *in vitro* dermal permeation assays should be minimized to the necessary level to attain the desired solubility or partition of the lipophilic compound tested;
- The effect of ethanol on the permeability properties of synthetic membranes (candidate skin models) should be further investigated for a range of compounds covering high and low lipophilic compounds to lay the groundwork for their potential acceptance in future methodologies.

CRediT authorship contribution statement

Dorinda Marques-da-Silva: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Margarida Franco:** Investigation. **Cristiana Violante:** Investigation. **Ricardo Lagoa:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2025.105931.

Data availability

Data will be made available on request.

References

Azarbayjani, A.F., Lin, H., Yap, C.W., Chan, Y.W., Chan, S.Y., 2010. Surface tension and wettability in transdermal delivery: a study on the in-vitro permeation of haloperidol with Cyclodextrin across human epidermis. J. Pharm. Pharmacol. 62 (6), 770–778. https://doi.org/10.1211/JPP.62.06.0014.

Beriro, D.J., Cave, M.R., Wragg, J., Thomas, R., Wills, G., Evans, F., 2016. A review of the current state of the art of physiologically-based tests for measuring human dermal in vitro bioavailability of polycyclic aromatic hydrocarbons (PAH) in soil. In: Journal of Hazardous Materials, vol.305. Elsevier B.V, pp. 240–259. https://doi.org/10.1016/j.jhazmat.2015.11.010.

Berner, B., Mazzenga, G.C., Otte, J.H., Steffens, R.J., Juang, R.-H., Ebert, C.D., 1989. Ethanol: water mutually enhanced transdermal therapeutic system II: skin permeation of ethanol and nitroglycerin. J. Pharmaceut. Sci. 78 (5), 402–407. https://doi.org/10.1002/JPS.2600780512.

Bommannan, D., Potts, R.O., Guy, R.H., 1991. Examination of the effect of ethanol on human stratum corneum in vivo using infrared spectroscopy. J. Contr. Release 16 (3), 299–304. https://doi.org/10.1016/0168-3659(91)90006-Y.

Chedik, L., Baybekov, S., Cosnier, F., Marcou, G., Varnek, A., Champmartin, C., 2024. An update of skin permeability data based on a systematic review of recent research. Scientific Data 2024 11 (1), 1–14. https://doi.org/10.1038/s41597-024-03026-4, 11 (1).

Coelho, C.D.F., Paiva, V.S., Almeida, Z.L., Jesus, J.A., Marteleira, M., Ramos, C.V., Cruz, P.F., Costa, T., Moura, C.S., Trindade, D., Brito, R.M.M., Lagoa, R., Vaz, D.C., Moreno, M.J., 2025. Serum-PEG and BSA-PEG hydrogels as advanced platforms for evaluating plasma protein binding. Mater. Today Chem. 45, 102565. https://doi.org/10.1016/J.MTCHEM.2025.102565.

Colas, A., Curtis, J., 2013. Silicones. In: Biomaterials Science: an Introduction to Materials, third ed. Academic Press, pp. 82–91. https://doi.org/10.1016/B978-0-08-087780-8.00010-3.

EPA, 2020. Chlorpyrifos: Third Revised Human Health Risk Assessment for Registration Review. United States Environmental Protection Agency. https://www.regulations.gov/document/EPA-HQ-OPP-2008-0850-0944.

Farahat, F.M., Fenske, R.A., Olson, J.R., Galvin, K., Bonner, M.R., Rohlman, D.S., Farahat, T.M., Lein, P.J., Anger, W.K., 2010. Chlorpyrifos exposures in Egyptian cotton field workers. Neurotoxicology 31 (3), 297–304. https://doi.org/10.1016/j.neuro.2010.02.005.

Fenske, R.A., Elkner, K.P., 1990. Multi-route exposure assessment and biological monitoring of urban pesticide applicators during structural control treatments with chlorpyrifos. Toxicol. Ind. Health 6 (3–4), 349–371. https://doi.org/10.1177/074823379000600301.

Franz, T.J., 1975. Percutaneous absorption. On the relevance of in vitro data. J. Invest. Dermatol. 64, 190–195. https://doi.org/10.1111/1523-1747.ep12533356.

Griffin, P., Mason, H., Heywood, K., Cocker, J., 1999. Oral and dermal absorption of chlorpyrifos: a human volunteer study. Occup. Environ. Med. 56 (1), 10–13. https://doi.org/10.1136/oem.56.1.10.

Griffin, P., Payne, M., Mason, H., Freedlander, E., Curran, A.D., Cocker, J., 2000. The in vitro percutaneous penetration of chlorpyrifos. Hum. Exp. Toxicol. 19 (2), 104–107. https://doi.org/10.1191/096032700678815684.

Gupta, R., Badhe, Y., Rai, B., Mitragotri, S., 2020. Molecular mechanism of the skin permeation enhancing effect of ethanol: a molecular dynamics study. RSC Adv. 10 (21), 12234–12248. https://doi.org/10.1039/D0RA01692F.

Haq, A., Dorrani, M., Goodyear, B., Joshi, V., Michniak-Kohn, B., 2018. Membrane properties for permeability testing: skin versus synthetic membranes. Int. J. Pharm. 539 (1–2), 58–64. https://doi.org/10.1016/j.ijpharm.2018.01.029.

Haq, A., Michniak-Kohn, B., 2018. Effects of solvents and penetration enhancers on transdermal delivery of thymoquinone: permeability and skin deposition study. Drug Deliv. 25 (1), 1943. https://doi.org/10.1080/10717544.2018.1523256.

Hatta, I., Ohta, N., Nakazawa, H., 2017. A possible percutaneous penetration pathway that should be considered. Pharmaceutics 9 (3), 26. https://doi.org/10.3390/PHARMACEUTICS9030026.

Hayat, K., Afzal, M., Aqueel, M.A., Ali, S., Saeed, M.F., Qureshi, A., Ullah, M., 2019. Insecticide toxic effects and blood biochemical alterations in occupationally exposed individuals in Punjab, Pakistan. Sci. Total Environ. 655, 102–111. https://doi.org/10.1016/j.scitotenv.2018.11.175.

Hopf, N.B., Champmartin, C., Schenk, L., Berthet, A., Chedik, L., Du Plessis, J.L., Franken, A., Frasch, F., Gaskin, S., Johanson, G., Julander, A., Kasting, G., Kilo, S., Larese Filon, F., Marquet, F., Midander, K., Reale, E., Bunge, A.L., 2020. Reflections on the OECD guidelines for in vitro skin absorption studies. Regul. Toxicol. Pharmacol. 117, 104752. https://doi.org/10.1016/j.yrtph.2020.104752.

Hopf, N.B., Spring, P., Hirt-Burri, N., Jimenez, S., Sutter, B., Vernez, D., Berthet, A., 2018. Polycyclic aromatic hydrocarbons (PAHs) skin permeation rates change with simultaneous exposures to solar ultraviolet radiation (UV-S). Toxicol. Lett. 287, 122–130. https://doi.org/10.1016/j.toxlet.2018.01.024.

Keunchkarian, S., Reta, M., Romero, L., Castells, C., 2006. Effect of sample solvent on the chromatographic peak shape of analytes eluted under reversed-phase liquid chromatogaphic conditions. J. Chromatogr. A 1119 (1–2), 20–28. https://doi.org/10.1016/J.CHROMA.2006.02.006.

Kluxen, F.M., Totti, S., Maas, W., Toner, F., Page, L., Webbley, K., Nagane, R., Mingoia, R., Whitfield, C., Kendrick, J., Valentine, C., Dorange, J.B., Egron, C., Imart, C., Domoradzki, J.Y., Fisher, P., Lorez, C., McEuen, S., Felkers, E., et al., 2022. An OECD TG 428 study ring trial with 14C-Caffeine demonstrating repeatability and robustness of the dermal absorption in vitro method. Regul. Toxicol. Pharmacol. 132, 105184. https://doi.org/10.1016/J.YRTPH.2022.105184.

Kovács, A., Zsikó, S., Falusi, F., Csányi, E., Budai-Szűcs, M., Csóka, I., Berkó, S., 2021. Comparison of synthetic membranes to heat-separated human epidermis in skin permeation studies in vitro. Pharmaceutics 13 (12), 2106. https://doi.org/10.3390/PHARMACEUTICS13122106.

Kovalev, A.E., Dening, K., Persson, B.N.J., Gorb, S.N., 2014. Surface topography and contact mechanics of dry and wet human skin. Beilstein J. Nanotechnol. 5 (1), 1341–1348. https://doi.org/10.3762/BJNANO.5.147, 147, 5.

Kwak, S., Brief, E., Langlais, D., Kitson, N., Lafleur, M., Thewalt, J., 2012. Ethanol perturbs lipid organization in models of stratum corneum membranes: an investigation combining differential scanning calorimetry, infrared and 2H NMR spectroscopy. Biochim. Biophys. Acta Biomembr. 1818 (5), 1410–1419. https://doi.org/10.1016/J.BBAMEM.2012.02.013.

Li, A., Yalkowsky, S.H., 1994. Solubility of organic solutes in ethanol/water mixtures. J. Pharmaceut. Sci. 83 (12), 1735–1740. https://doi.org/10.1002/JPS.2600831217.

Lian, G., Chen, L., Han, L., 2008. An evaluation of mathematical models for predicting skin permeability. J Pharm Sci 97, 584–598. https://doi.org/10.1002/jps.21074.

Marques-da-Silva, D., Lagoa, R., 2022. Synthetic membranes as an alternative to animal skin to investigate dermal permeation of chlorpyrifos. Med. Sci. Forum 11 (1), 3. https://doi.org/10.3390/BiTaP-12786.

Marques-da-Silva, D., Lopes, J.M., Correia, I., Silva, J.S., Lagoa, R., 2022. Removal of hydrophobic organic pollutants and copper by alginate-based and polycaprolactone materials. Processes 10 (11), 2300. https://doi.org/10.3390/pr10112300.

Merck, 2018. Strat-M membrane brochure. https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/marketing/global/documents/201/563/strat-m-membrane-br2545en-mk.pdf.

Mitragotri, S., Anissimov, Y.G., Bunge, A.L., Frasch, H.F., Guy, R.H., Hadgraft, J., Kasting, G.B., Lane, M.E., Roberts, M.S., 2011. Mathematical models of skin permeability: an overview. Int. J. Pharm. 418 (1), 115–129. https://doi.org/10.1016/j.ijpharm.2011.02.023.

Moore, C.A., Wilkinson, S.C., Blain, P.G., Dunn, M., Aust, G.A., Williams, F.M., 2014. Percutaneous absorption and distribution of organophosphates (chlorpyrifos and dichlorvos) following dermal exposure and decontamination scenarios using in vitro human skin model. Toxicol. Lett. 229 (1), 66–72. https://doi.org/10.1016/j.toxlet.2014.06.008.

Neupane, R., Boddu, S.H.S., Renukuntla, J., Babu, R.J., Tiwari, A.K., 2020. Alternatives to biological skin in permeation studies: current trends and possibilities. Pharmaceutics 12 (2), 152. https://doi.org/10.3390/PHARMACEUTICS12020152.

Nolan, R.J., Rick, D.L., Freshour, N.L., Saunders, J.H., 1984. Chlorpyrifos: pharmacokinetics in human volunteers. Toxicol. Appl. Pharmacol. 73 (1), 8–15. https://doi.org/10.1016/0041-008X(84)90046-2.

OECD, 2004. Guidance Document for the Conduct of Skin Absorption Studies. Series on Testing and Assessment, No 28. Organisation for Economic Co-operation and Development. https://doi.org/10.1787/9789264078796-en.

OECD, 2022. Guidance Notes on Dermal Absorption Studies - Series on Testing and Assessment No. 156, second ed. Organisation for Economic Co-Operation and Development https://one.oecd.org/document/ENV/JM/MONO(2011)36/REV1/en/pdf.

Olkowska, E., Gržinić, G., 2022. Skin models for dermal exposure assessment of phthalates. Chemosphere 295, 133909. https://doi.org/10.1016/j.chemosphere.2022.133909.

Panuwet, P., Prapamontol, T., Chantara, S., Thavornyuthikarn, P., Montesano, M.A., Whitehead, R.D., Barr, D.B., 2008. Concentrations of urinary pesticide metabolites in small-scale farmers in Chiang Mai province, Thailand. Sci. Total Environ. 407 (1), 655–668. https://doi.org/10.1016/j.scitotenv.2008.08.044.

Pershing, L.K., Lambert, L.D., Knutson, K., 1990. Mechanism of ethanol-enhanced estradiol permeation across human skin in vivo. Pharm. Res. 7 (2), 170–175. https://doi.org/10.1023/A:1015832903398.

Raykar, P.V., Fung, M.C., Anderson, B.D., 1988. The role of protein and lipid domains in the uptake of solutes by human stratum corneum. Pharm. Res. 5 (3), 140–150. https://doi.org/10.1023/A:1015956705293.

Sartorelli, P., Aprea, C., Cenni, A., Novelli, M.T., Orsi, D., Palmi, S., Matteucci, G., 1998. Prediction of percutaneons absorption from physicochemical data: a model based on data of in vitro experiments. Ann. Occup. Hyg. 42 (4), 267–276. https://doi.org/10.1016/S0003-4878(98)00021-0.

SCCS, 2010. The Scientific Committee on Consumer Safety Basic Criteria for the in Vitro Assessment of Dermal Absorption of Cosmetic Ingred | Enhanced Reader.

Silva, J., Marques-da-Silva, D., Lagoa, R., 2021a. Reassessment of the experimental skin permeability coefficients of polycyclic aromatic hydrocarbons and organophosphorus pesticides. Environ. Toxicol. Pharmacol. 86, 103671. https://doi.org/10.1016/j.etap.2021.103671.

Silva, J., Marques-da-Silva, D., Lagoa, R., 2021b. Towards the development of delivery systems of bioactive compounds with eyes set on pharmacokinetics. Modeling and Control of Drug Delivery Systems 125–144. https://doi.org/10.1016/B978-0-12-821185-4.00006-3.

Simon, A., Amaro, M.I., Healy, A.M., Cabral, L.M., de Sousa, V.P., 2016. Comparative evaluation of rivastigmine permeation from a transdermal system in the franz cell using synthetic membranes and pig ear skin with in vivo-in vitro correlation. Int. J. Pharm. 512 (1), 234–241. https://doi.org/10.1016/j.ijpharm.2016.08.052.

Sung, C.R., Kim, K.B., Lee, J.Y., Lee, B.M., Kwack, S.J., 2019. Risk assessment of ethylhexyl dimethyl PABA in cosmetics. In: Toxicological Research, vol. 35. Korean Society of Toxicology, pp. 131–136. https://doi.org/10.5487/TR.2019.35.2.131, 2.

Tan, Y., Yao, J., Zhu, H.P., 2020. Effects of ethanol content on the properties of silicone rubber foam. J. Polym. Eng. 40 (7), 543–550. https://doi.org/10.1515/polyeng-2019-0205.

Thors, L., Koch, B., Koch, M., Hägglund, L., Bucht, A., 2016. In vitro human skin penetration model for organophosphorus compounds with different physicochemical properties. Toxicol. Vitro 32, 198–204. https://doi.org/10.1016/j.tiv.2016.01.003.

Uchida, T., Kadhum, W.R., Kanai, S., Todo, H., Oshizaka, T., Sugibayashi, K., 2015. Prediction of skin permeation by chemical compounds using the artificial membrane, Strat-M™. Eur. J. Pharmaceut. Sci. 64, 1338–1346. https://doi.org/10.1016/j.ejps.2014.11.002.

Uchida, T., Yakumaru, M., Nishioka, K., Higashi, Y., Sano, T., Todo, H., Sugibayashi, K., 2016. Evaluation of a silicone membrane as an alternative to human skin for determining skin permeation parameters of chemical compounds. Chem. Pharm. Bull. 64, 1338–1346. https://doi.org/10.1248/cpb.c16-00322.

University of Canterbury. (n.d.). Determination of ethanol concentration in aqueous solutions. Retrieved November 26, 2024, from https://www.canterbury.ac.nz/content/dam/uoc-main-site/documents/pdfs/d-other/Determination-of-Ethanol-Concentration-in-Aqueous-Solutions.pdf.

Van Der Merwe, D., Riviere, J.E., 2005. Comparative studies on the effects of water, ethanol and water/ethanol mixtures on chemical partitioning into Porcine stratum corneum and silastic membrane. Toxicol. Vitro 19 (1), 69–77. https://doi.org/10.1016/J.TIV.2004.06.002.

Waheed, S., Halsall, C., Sweetman, A.J., Jones, K.C., Malik, R.N., 2017. Pesticides contaminated dust exposure, risk diagnosis and exposure markers in occupational and residential settings of lahore, Pakistan. Environ. Toxicol. Pharmacol. 56, 375–382. https://doi.org/10.1016/j.etap.2017.11.003.

Watkinson, R.M., Herkenne, C., Guy, R.H., Hadgraft, J., Oliveira, G., Lane, M.E., 2009. Influence of ethanol on the solubility, ionization and permeation characteristics of ibuprofen in silicone and human skin. Skin Pharmacol. Physiol. 22 (1), 15–21. https://doi.org/10.1159/000183922.